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Diagnosis of Enterovirus and Rhinovirus Infections by RT-PCR and Time-Resolved Fluorometry With Lanthanide Chelate Labeled Probes

M. Lönnrot,[1]* M. Sjöroos,[2] K. Salminen,[3] M. Maaronen,[3] T. Hyypiä,[3,4] and H. Hyöty[1,3]

[1]*Department of Virology, Medical School and University Hospital of Tampere University, Tampere, Finland*
[2]*Department of Biochemistry and Biotechnology, University of Turku, Turku, Finland*
[3]*Department of Virology, University of Turku, Turku, Finland*
[4]*Haartman Institute, Department of Virology, University of Helsinki, Helsinki, Finland*

Detection of enteroviruses and rhinoviruses has traditionally been based on laborious and time-consuming virus isolation. Recently, rapid and sensitive assays for detecting enterovirus and rhinovirus genomic sequences by reverse transcription-polymerase chain reaction (RT-PCR) have been introduced. An RT-PCR assay is described that amplifies both enteroviral and rhinoviral sequences, followed by liquid-phase hybridization carried out in a microtiter plate format. In the hybridization assay, amplicons are identified by enterovirus- or rhinovirus-specific probes carrying lanthanide chelate labels, which can be detected simultaneously by time-resolved fluorometry. The sensitivity and specificity of the RT-PCR-hybridization method were evaluated with a representative collection of enteroviruses and rhinoviruses and tested further its applicability to the clinical setting with cerebrospinal fluid samples and nasopharyngeal aspirates. The RT-PCR assay amplified all enteroviruses and rhinoviruses tested, and all but one amplicon gave a positive result in the subsequent hybridization assay. The RT-PCR-hybridization method was more sensitive than virus isolation for the detection of enteroviruses and rhinoviruses in the clinical samples. High sensitivity, rapidity, and easy performance make the assay suitable for the routine diagnosis of enterovirus and rhinovirus infections. ***J. Med. Virol. 59:378–384, 1999.***

KEY WORDS: picornavirus; meningitis; respiratory infection

INTRODUCTION

Enteroviruses and rhinoviruses are common causes of human disease, the former being responsible for such conditions as aseptic meningitis and myocarditis [Morens and Pallansch, 1995], and the latter known as the most frequent cause of the common cold [Mäkelä et al., 1998]. The diagnosis of enterovirus infections has traditionally had more clinical significance than that of rhinovirus infections due to the more severe forms of enterovirus diseases requiring hospitalization. However, new data have broadened the spectrum of diseases with rhinovirus association: in addition to common colds, rhinoviruses can cause or predispose to otitis media [Pitkäranta et al.,1998; Chonmaitree et al., 1992], sinusitis [Gwaltney et al., 1994; Pitkäranta et al., 1997], and exacerbations of asthma [Nicholson et al., 1993; Johnston et al., 1995], and they can also cause lower respiratory tract diseases in infants and young children [Schmidt and Fink, 1991; McMillan et al., 1993] and in elderly adults [Nicholson et al., 1997]. Thus, there is an increasing need for diagnostic tools to detect rhinovirus infection.

Until recently, isolation in culture has been the only method to detect enteroviruses and rhinoviruses in clinical specimens. Isolated enteroviruses are usually typed by a panel of neutralizing antisera, whereas rhinoviruses are identified by an acid lability test. New methods for diagnostic purposes are actively being developed, because traditional culture-based methods are time-consuming, laborious and have only moderate sensitivity. Moreover, all enteroviruses do not grow in cell cultures, which further decreases the sensitivity of virus isolation. In addition to virus isolation, serological tests are used for diagnosis of enterovirus infections. Serology is complicated, however, by the large number of enterovirus serotypes as well as by individual variations in serological responses.

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*Correspondence to: Maria Lönnrot, Department of Virology, University of Tampere Medical School, PO Box 607, FIN-33101 Tampere, Finland. E-mail: maria.lonnrot@uta.fi

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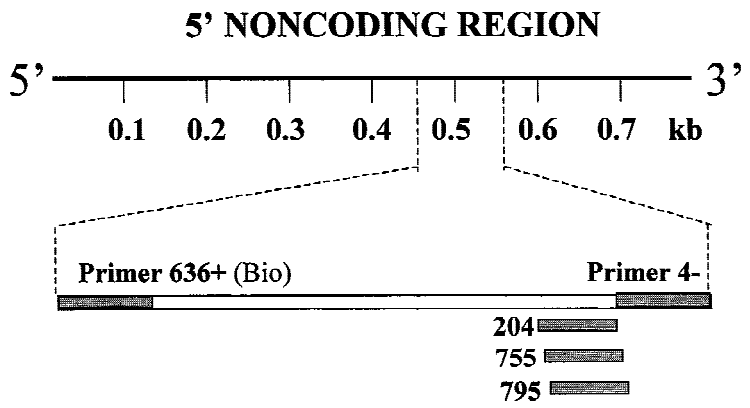

OLIGONUCLEOTIDE	SEQUENCE	MAP-POSITION
Primer 636+ (Bio)	5'-CGGCCCCTGAATGCGGCTAA-3'	454-473[1]
Primer 4-	5'-GAAACACGGACACCCAAAGTA-3'	548-568[1]
Probe 204 (rhino)	5'-TAGTTGGTCCCITCCCG-3'	528-544[2]
Probe 755 (entero)	5'-TAITCGGTTCCGCTGC-3'	534-549[1]
Probe 795 (entero+rhino)	5'-AAAGTAGTIGGTICC-3'	539-553[1]

Fig. 1. Position of the amplified sequences in the 5′ noncoding region of the picornavirus genomes. Primer and probe sequences with map positions referring to the genomes of [1]echovirus 11 [Dahllund et al., 1995] and [2]human rhinovirus 1B [Hughes et al., 1988] are shown.

The weaknesses of the traditional methods used in laboratory diagnostics of enterovirus and rhinovirus infections have been largely overcome recently as sensitive and rapid methods based on the detection of enteroviral and rhinoviral genomic sequences have been introduced [Hyypiä et al., 1989; Andreoletti et al., 1996; Arola et al., 1996; Halonen et al., 1995; Sawyer et al., 1994; Muir et al., 1993; Johnston et al., 1993; Ireland et al, 1993; Arruda and Hayden, 1993;. Zoll et al., 1992; Rotbart, 1990; Hyypiä et al., 1998]. There is, however, an obvious need to develop the method further to improve its suitability in clinical laboratories. A reverse transcription-polymerase chain reaction (RT-PCR) assay is described that amplifies both enterovirus and rhinovirus sequences, accompanied by a liquid-phase hybridization assay in a microtiter plate format. The hybridization assay uses time-resolved fluorometry, which has been applied previously for detection of PCR-amplified virus sequences [Halonen et al., 1995; Hierholzer et al., 1993; Dahlen et al., 1991]. This method clearly has higher sensitivity than virus isolation; the distinction between enterovirus and rhinovirus sequences is simple. The method is rapid and easy to perform, and the microtiter plate format permits automation of the hybridization assay. Thus, the method is suitable in routine diagnostics of enterovirus and rhinovirus infections.

MATERIALS AND METHODS

Virus Stocks

Prototype enteroviruses and rhinoviruses were obtained from the American Type Culture Collection, and virus isolates from the collection at the Department of Virology, University of Turku. Enteroviruses were grown in susceptible cells and typed using World Health Organization (WHO) serum pools A to H. Rhinoviruses were grown in HeLa cells and identified on the basis of their acid lability.

Clinical Specimens

All clinical specimens were collected from patients living within the district of Turku University Hospital in southwestern Finland and sent for testing as part of the daily virological diagnostic routine. The specimens consisted of 81 cerebrospinal fluid (CSF) samples and 69 nasopharyngeal aspirates (NPA) from patients with signs and symptoms of meningitis/encephalitis or respiratory infection, respectively, during 1996 and 1997.

Virus Isolation

Virus isolation was done by using the Ohio strain of HeLa cells, LLC-Mk_2 cells, A549 cells, and human foreskin fibroblasts according to the routine procedures. Supernatants of cell cultures exhibiting cytopathogenic effect were tested by neutralizing antisera and an acid lability test.

Oligonucleotides

Primers and probes were selected from the 5′ noncoding region (NCR) of the picornavirus genome (Fig. 1). Primers producing an approximately 120-bp amplicon were selected from conserved regions in enteroviruses and rhinoviruses [Hyypiä et al., 1989]. The selection of primers was based on RT-PCR assays described previously for enteroviruses and rhinoviruses. The negative-strand primer (4–) has the same map position as previously described enterovirus probe [Rotbart, 1990] and primer 4+ [Arola et al., 1996]. The positive-

strand primer (636+) is located in the same genomic region as several other primers described previously for detection of enteroviruses [Arola et al., 1996; Halonen et al., 1995; Zoll et al., 1992; Chapman et al., 1990; Rotbart, 1990]. The primer pair has also shown to be highly sensitive for detection of rhinoviruses [Hyypiä et al., 1998; Santti et al., 1997]. The 5′ end of the positive-strand primer was biotinylated (CyberGene, Uddingen, Sweden). Probes with intra-genus conservation but maximum difference between enteroviruses and rhinoviruses (entero probe 755 and rhino probe 204) as well as a probe with maximum homology between the two genera (entero+rhino probe 795) were chosen on the basis of the alignment of the known sequences. Probe 204 has been described earlier [Halonen et al., 1995]. The 5′ ends of these probes were labeled with lanthanide chelates of europium (Eu) and samarium (Sm) as described earlier [Dahlen et al., 1991].

RT-PCR

RNA was extracted from 140 μl of CSF and NPA samples using QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The reverse transcriptase (RT) reaction (total volume 40 μl) contained 10 μl extracted sample RNA, RT-buffer (Promega, Madison,WI), 0.5 mM deoxynucleoside triphosphates (Pharmacia Biotech, Uppsala, Sweden), 4 U of RNase inhibitor (Promega), 50 pmol of the negative-strand primer (4–) and 20 U of Moloney murine leukemia virus reverse transcriptase enzyme (Promega). After incubation for 60 min at 37°C, 10 μl of the cDNA reaction mixture was added to 90 μl of the PCR reaction mixture containing PCR-buffer (DyNAzyme, Finnzymes, Espoo, Finland), 0.2 mM deoxynucleoside triphosphates (Pharmacia Biotech), 20 pmol of both negative-strand (4–) and positive-strand (Bio-636+) primers and 1 U of DNA polymerase enzyme (DyNAzyme). Two drops of mineral oil were added, and the tubes were incubated in a DNA Thermal Cycler (Perkin-Elmer, Cetus Corp., Norwalk, CT), first for 3 min at 94°C, then through 40 cycles of programmed amplification (denaturation, 94°C, 30 sec; annealing, 53°C, 45 sec; extension, 72°C, 1 min) and finally for 7 min at 72°C. Separate rooms were used for each step of the RT-PCR work. Every assay included samples of known enterovirus and rhinovirus serotypes as positive controls as well as several negative control samples.

Liquid-Phase Hybridization Assay

The assay principle (Fig. 2) has been previously described [Sjöroos et al., 1995]. A total of 10 μl of the amplification product and 50 μl of DELFIA® Assay Buffer (EG & G® Wallac Oy, Turku, Finland) were added into streptavidin-coated microtiter wells (DELFIA), and incubated at room temperature with shaking for 30 min to collect the amplified DNA onto the wells. The wells were washed at room temperature with DELFIA Wash Solution in an automated platewasher. The bound DNA was denatured by adding 150

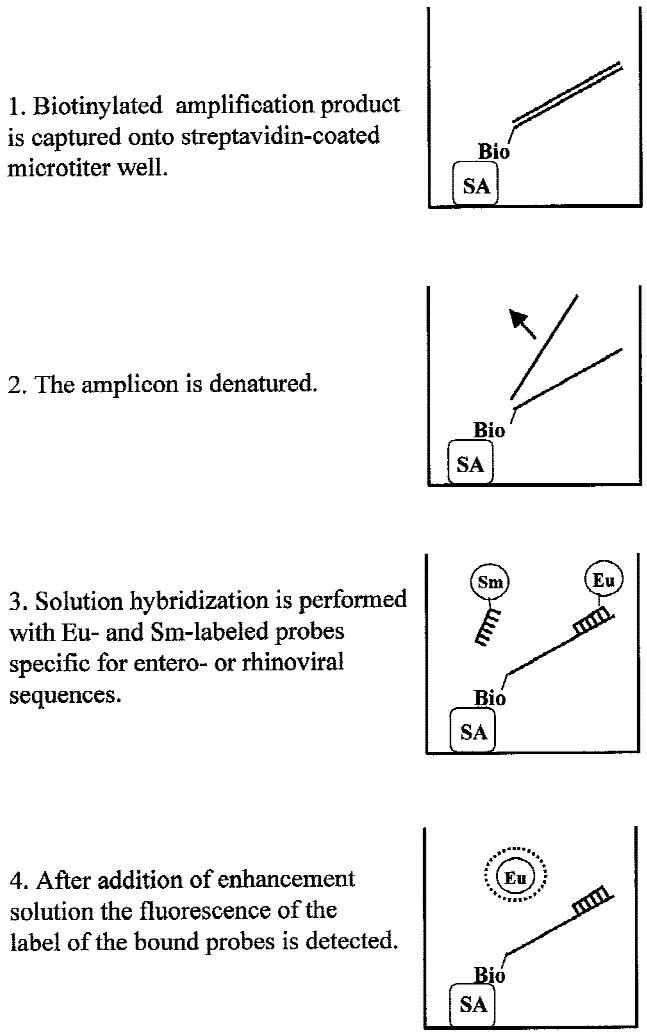

Fig. 2. The principle of the liquid hybridization assay with an enteroviral RT-PCR amplicon.

μl 50 mM NaOH and shaking for 5 min at room temperature. The nonbiotinylated strand was removed by washing as described previously. Bound DNA was detected by hybridization using an Eu-labeled probe 755 and a Sm-labeled probe 204 in the same solution. In addition, we used a parallel hybridization with Eu-labeled probe 795. Each probe was used at a concentration of 30 ng/ml in DELFIA Assay Buffer, supplemented with 0.85 M NaCl and 1% Tween. A total of 100 μl of hybridization mixture was added per well, and the reaction was carried out for 2 hr at 37°C (probes 755 and 204) or at room temperature (probe 795). Unbound probes were washed away at 45°C (probes 755 and 204) or at room temperature (probe 795) using DELFIA Wash Solution. For the detection of the Eu and Sm signals of the bound probes, 200 μl of DELFIA Enhancement Solution was added into each well. After shaking for 25 min at room temperature, time-resolved fluorescence was measured using a VICTOR™1420 Multilabel Fluorometer (EG & G Wallac Oy).

RESULTS

Development of the Hybridization Assay

RT-PCR amplicons from reference viruses and virus isolates were used for the optimization of the hybridization assay. Out of 12 different candidate probes, three were selected for the final optimization experiments. One of these detected preferentially enterovirus sequences (755), one rhinovirus sequences (204), and one both enterovirus and rhinovirus sequences (795). The conditions for each step in the hybridization assay were optimized. Optimal hybridization conditions differed considerably between the three probes with regard to the temperatures of hybridization and the subsequent washing. Probe 755 required a hybridization temperature as high as 35–40°C for optimal reactivity and the optimal temperature in the subsequent washing was 45°C, whereas the signal obtained with the probe 795 decreased markedly in hybridization temperatures above 30°C. Probe 204 was less temperature sensitive, as it worked moderately well in temperatures of 20–45°C, with an optimal hybridization temperature of 35–40°C. Thus, probe 204 can be combined with either probe 755 or 795. The signal intensity of 130 clinical samples negative both in agarose gel electrophoresis and in hybridization was as low as the background signal intensity obtained with negative control samples, in which water was used as template (mean signal-to-background ratio approximately 1, range 0.3–2.2). The cut-off value for a positive specimen was determined to be five times the background value obtained with negative controls included in each assay.

Identification of Amplified Sequences in Prototype Viruses and Clinical Isolates

Amplification in RT-PCR was demonstrated by the presence of a fragment of expected molecular weight in agarose gel electrophoresis. All available enterovirus and rhinovirus serotypes were found to be amplified by the primers. Binding of the three probes was tested using RT-PCR amplicons from prototype strains and clinical isolates representing altogether 30 different enterovirus serotypes, nine different rhinovirus serotypes and 20 clinical rhinovirus isolates of unknown serotypes. Table I summarizes these results, indicating that this combination of probes was able to detect amplified sequences from all but one rhinovirus isolate. Probe 755 hybridized with all enterovirus sequences and one rhinovirus sequence. The binding of probe 204 was specific for rhinovirus sequences, but it failed to detect 5 of the 20 untyped rhinoviruses. However, these sequences were detected by the third probe 795, which bound to 52 of a total of 59 sequences from both enteroviruses and rhinoviruses. Different intensity in the binding of probes to sequences from enteroviruses and rhinoviruses permitted clear distinction between these virus groups.

Sensitivity of RT-PCR Hybridization Assay

The sensitivity of the RT-PCR-hybridization assay was examined by testing a dilution series of enterovirus RNA (Fig. 3). The RNA was extracted from purified CBV4 by using proteinase K-sodium dodecyl sulfate treatment, followed by phenol extraction and ethanol precipitation. Concentration of the extracted RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer. In the hybridization assay, the RT-PCR amplicon corresponding to 0.015 fg of RNA gave a positive signal. In gel electrophoresis, a very weak band corresponding to 0.15 fg RNA was visible but practically only amplicons corresponding to 1.5 fg RNA were visible.

The sensitivity of the RT-PCR-hybridization assay was also compared with that of virus isolation in the examination of clinical samples (Table II). Altogether 81 CSF and 69 NPA specimens from the routine diagnostic material were tested using both virus isolation in culture and the RT-PCR hybridization assay. Enterovirus was isolated from seven CSF specimens and from two NPA specimens. Enterovirus RNA was found in all these specimens as well as in 19 CSF and 8 NPA specimens. Rhinovirus was isolated from six NPA specimens and detected by RT-PCR in all of these and in an additional 10 samples. Furthermore, four NPA specimens, which were negative by virus isolation, proved positive by hybridization only with the enterorhinovirus probe 795, indicating either enterovirus or rhinovirus infection.

DISCUSSION

Clinical laboratory diagnosis of enterovirus and rhinovirus infections has significantly improved recently, as new methods based on virus RNA detection using RT-PCR have been developed [Hyypiä et al., 1989; Andreoletti et al., 1996; Arola et al., 1996; Halonen et al., 1995; Sawyer et al., 1994; Muir et al., 1993; Johnston et al., 1993; Ireland et al., 1993; Arruda and Hayden, 1993; Zoll et al., 1992; Rotbart, 1990; Hyypiä et al., 1998]. Most of these methods take advantage of the 5′ NCR which is highly conserved among enteroviruses and rhinoviruses, and therefore the assays are not entirely specific for only enteroviruses or rhinoviruses. This nonspecificity is of minor importance if, for example, only CSF samples are studied, as rhinoviruses have never been isolated from CSF; however, in the case of nasal or pharyngeal specimens, it is a problem, and hybridization or sequencing of the amplification products is required to ensure a specific result. Our assay uses primers that have been used for detection of both enteroviruses [Arola et al., 1996; Halonen et al., 1995; Zoll et al., 1992; Chapman et al., 1990; Rotbart, 1990] and rhinoviruses [Hyypiä et al., 1998; Santti et al., 1997], and seem to be highly sensitive among picornaviral primers [Hyypiä et al., 1998; Santti et al., 1997]. The picornavirus genome is highly conserved at the primer locations, but fortunately differences between the amplified enterovirus and rhinovirus se-

TABLE I. Signal-to-Background Ratios Obtained With the Three Probes in Hybridization Assays With Amplicons Representing Different Enteroviruses and Rhinoviruses*

	Probe 755		Probe 204		Probe 795	
	Result +/–	Signal/background	Result +/–	Signal/background	Result +/–	Signal/background
Polio 1	+	52	–	1	+	8
Polio 2	+	15	–	2	+	19
Polio 3	+	98	–	1	+	28
Echo 1	+	>100	–	1	+	56
Echo 2	+	75	–	1	+	34
Echo 5	+	90	–	1	+	>100
Echo 6	+	>100	–	2	+	58
Echo 8	+	>100	–	1	+	35
Echo 13	+	75	–	1	+	26
Echo 14	+	50	–	1	+	72
Echo 15	+	80	–	1	+	38
Echo 16	+	22	–	2	+	7
Echo 20	+	>100	–	1	+	14
Echo 25	+	>100	–	1	+	>100
Echo 26	+	>100	–	1	+	27
Echo 27	+	>100	–	1	–	4
Echo 30	+	>100	–	1	+	48
Echo 31	+	>100	–	1	+	9
Echo 32	+	>100	–	1	–	2
Echo 33	+	>100	–	1	+	23
CAV 9	+	62	–	1	–	4
CAV 14	+	>100	–	1	+	43
CAV 16	+	>100	–	1	+	11
CAV 21	+	>100	–	1	+	33
CBV 2	+	>100	–	1	+	22
CBV 3	+	>100	–	3	+	36
CBV 4	+	>100	–	2	+	59
CBV 5	+	>100	–	2	+	13
CBV 6	+	>100	–	1	+	60
Entero 70	+	26	–	1	+	12
Rhino 1b	–	2	+	75	–	3
Rhino 3	–	5	+	>100	+	57
Rhino 9	–	2	+	>100	+	28
Rhino 11	–	2	+	>100	+	62
Rhino 13	+	13	+	>100	+	31
Rhino 14	–	3	+	>100	+	28
Rhino 29	–	1	+	>100	+	>100
Rhino 36	–	1	+	83	+	35
Rhino 48	–	1	+	60	–	4
Rhino, untyped	–	1	+	22	+	24
Rhino, untyped	–	1	+	19	+	79
Rhino, untyped	–	1	–	2	–	2
Rhino, untyped	–	1	+	13	+	13
Rhino, untyped	–	1	+	31	+	88
Rhino, untyped	–	1	+	12	+	14
Rhino, untyped	–	1	+	31	+	>100
Rhino, untyped	–	2	+	17	+	22
Rhino, untyped	–	1	+	36	–	4
Rhino, untyped	–	1	+	58	+	40
Rhino, untyped	–	1	–	1	+	>100
Rhino, untyped	–	1	+	21	+	19
Rhino, untyped	–	1	+	40	+	89
Rhino, untyped	–	1	+	38	+	95
Rhino, untyped	–	1	+	14	+	84
Rhino, untyped	–	1	+	52	+	31
Rhino, untyped	–	1	–	1	+	>100
Rhino, untyped	–	1	–	1	+	>100
Rhino, untyped	–	1	–	1	+	>100
Rhino, untyped	–	1	+	19	+	56

*Signal-to-background value 5 was used as cutoff limit for positivity. Note horizontal evaluation of signal intensities in case of each serotype, but vertical comparisons between different serotypes are hampered by varying amounts of PCR amplification product.

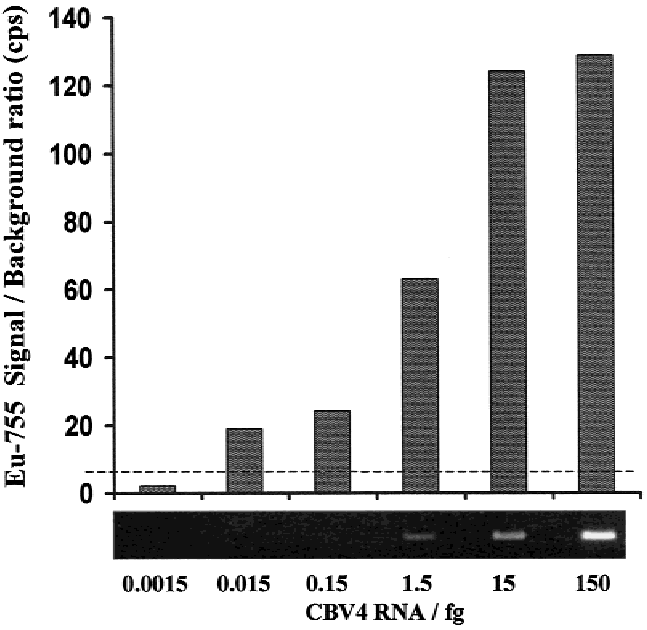

Fig. 3. Sensitivity of the RT-PCR assay accompanied by liquid hybridization with enterovirus probe 755 was tested with a dilution series of RNA extracted from purified CBV4. The RNA amounts used in the reverse transcription are indicated in the x-axis; corresponding hybridization result with Eu-labeled probe 755 is shown with *bars. Dashed line,* signal-to-background value 5, which was used as cutoff limit for positivity. Result of agarose gel electrophoresis with the same amplicons is shown for comparison; 10 and 15 μl of each RT-PCR amplicon were used in liquid hybridization and gel electrophoresis, respectively.

quences allow subsequent differentiation of the amplicons in hybridization with specific probes.

Sensitivity achieved by the RT-PCR-hybridization assay was found to be considerably higher than the sensitivity of virus isolation (Table II). The sensitivity is also high in comparison with previously described enterovirus RT-PCR assays; for example, in a recent European Union Quality Control Programme testing (Enterovirus Proficiency Panel) our assay specificity was 100% and the sensitivity was higher than the sensitivity of the Amplicor Enterovirus test by Roche Molecular Systems (0.01 vs. 0.1 $TCID_{50}$ using CAV 9; data not shown). High sensitivity together with rapid and easy performance speaks strongly in favor of RT-PCR-hybridization as a standard method in clinical diagnostics of enteroviruses and rhinoviruses. In the present study, the RT-PCR-hybridization was used only for testing of CSF and NPA specimens. However, the assay has high sensitivity with other clinical specimens as well, such as serum and stool (data not shown), which can be problematic as a result of PCR inhibitors.

Hybridization subsequent to RT-PCR is required for differentiation of the amplicons, because the amplified enterovirus and rhinovirus cDNA fragments are of approximately same length, producing identical bands in gel electrophoresis. The hybridization step also further increases the assay specificity and sensitivity as compared to gel electrophoresis (Fig. 3). The assay is rapid, as it takes only 4 hr, including <2 hr of actual hands-on time. Furthermore, performance in microtitration plate format provides an opportunity for bulk assays and automation.

Cross-reactivity between the enterovirus probe 755 and rhinovirus probe 204 was nearly nonexistent. Rhinovirus probe 204 did not hybridize to any of the tested enteroviral sequences. Enterovirus probe 755 gave a positive signal with only one of the tested rhinovirus serotypes (Table I), but in this case the result was suggestive of rhinovirus amplicon, as the difference between signal intensities of probes 755 and 204 was marked. However, the possibility of contamination of the stock rhinovirus with an enterovirus cannot be excluded. In seven cases, signal was obtained only by entero+rhinovirus probe 795 (Table I). These RT-PCR amplicons originated from NPA samples, implying that the amplified sequences may been either enteroviral or rhinoviral. However, the results of Table I suggest that these sequences may have been more likely rhinoviral, as the sensitivity of probe 755 seems excellent, whereas probe 204 fails to hybridize with some rhinoviral sequences. This finding also suggests that if only enteroviruses are to be detected, e.g., in CSF specimens, only probe 755 can be used, but accurate detection of rhinoviral sequences requires both probes 204 and 795.

The RT-PCR assay amplified sequences from all tested enterovirus and rhinovirus serotypes and isolates. Former enterovirus serotypes echovirus 22 and 23, currently known as parechoviruses 1 and 2, respectively, were not amplified by the RT-PCR (data not shown). This was expected due to the marked genetic differences between enteroviruses and parechoviruses [Stanway et al., 1994].

Isolated enteroviruses can usually be serotyped, but the RT-PCR-hybridization assay does not give a serotype-specific result. However, from a clinical point of view, differentiation between viral and bacterial disease, e.g., in patients with meningitis, is crucial and serotype information is of secondary importance. Because the typing procedure may take several weeks the result has usually only epidemiological interest. Discrimination between polio and nonpolio serotypes is one of the rare situations when enterovirus serotyping has clear clinical relevance. Sequence analysis of the RT-PCR amplicons has a potential to replace traditional serotyping in the future, as it gives detailed information that can be directly correlated with the increasing knowledge about the molecular determinants of pathogenesis of the virus strains.

The knowledge of diseases with rhinovirus association has recently expanded [Nicholson et al., 1993, 1997; Schmidt and Fink, 1991; McMillan et al., 1993; Johnston et al., 1995; Gwaltney et al., 1994; Pitkäranta et al., 1997, 1998; Chonmaitree et al., 1992]. This will probably affect the diagnosis of rhinovirus in the near future. The clinical relevance of rhinovirus diagnostics can be disputed in the case of the common cold, but in the differential diagnosis of otitis media, sinusitis, and lower respiratory infections, it may be useful.

The results show that the RT-PCR assay together

TABLE II. Comparison of the RT-PCR Hybridization Assay With Virus Isolation for Detection of Enteroviruses and Rhinoviruses in CSF and NPA Specimens

PCR hybrid result	Virus isolation result: CSF enterovirus +	CSF enterovirus–	NPA enterovirus +	NPA rhinovirus +	NPA entero– and rhinovirus–
Enterovirus +	7[a]	19[a]	2[a]	0	8[a]
Rhinovirus +	0	0	0	6[b]	10,[b] 4[c]
Negative	0	55	0	0	39

[a]Hybridization assay positive with enterovirus probe 755.
[b]Hybridization assay positive with rhinovirus probe 204.
[c]Hybridization assay positive only with enterovirus-rhinovirus probe 795, indicating either enterovirus or rhinovirus infection.

with a liquid hybridization assay can overcome the weaknesses of virus isolation: higher sensitivity together with rapid and easy analysis of large sample numbers are qualities that make the assay useful for the routine diagnosis of both enterovirus and rhinovirus infections. In addition, performance in a microtiter format makes the assay suitable for automation.

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